

4/27/99 - 5/24/99

4/27/99 - Subcloning GM-CSF and hSTNFR1 into pVet
• (His tag) Plasmid -

Our biggest difficulty is producing enough native STNFR1 to produce monoclonal Abs via immunization. Although we would prefer to use the leukryptic version of this protein, our 1929 transfectant (Clone 39) does not produce sufficient quantities. We will attempt at a later date, however, to purify the STNFR from the Hela sup^t that was AMS⁺ precipitated (pgs 5-9) - once we have a TNF affinity column. In the mean time, we will attempt to make native hSTNFR1 in *E. coli*. The protein has been produced previously in *E. coli* using the pet24a expression system (His-tag fusion proteins), but due to the extraordinary production of the recombinant protein, and due to the extensive secondary structure of STNFR1, much of the protein was found in inclusion bodies (i.e. much of the protein was denatured). We were not able to renature this protein. Sung, from Dr. Schweitzer's lab, proposed that if the origin of replication in these pet vectors was changed out for one of a low copy number plasmid, then the recombinant protein would be produced at levels conducive to retaining its native conformation. Therefore, he modified one of the pet vectors, pet156, such that the ColE1 origin of replication was exchanged for the pSC101 origin of replication from pWSK29. The T7 promoter, multiple cloning site, and other sequences from the original pet156 plasmid are conserved. This modified plasmid is now called pVet (4985bp).

We are going to subclone the hSTNFR1 gene, as well as the GM-CSF gene, previously cloned into pet24a into pVet, so that we will have a readily purifiable source of these proteins.

4/2.19

Started with glycerol stocks:

- neon's GmSF + pet24a in BL21(7) DE3 clone #6
 - My hSTNRT + pet 24a in BL21(7) DE3 clone #8
- Made 2 overnight cultures in LB + 25 µg/ml Kan

Made a glycerol stock of pVet in 211 Blue on 5/12/99

Made Diaga prep of lalk. Not pVet plasmid from Jung

Digest #1 with Xba I:

	Plasmid		
	GmSF	hSTNRT	pVet
sterile H ₂ O	33 µl	33 µl	1 µl
10x Buffer (Realt 2)	5 µl	5 µl	10 µl
DNA (1 µg)	10 µl	10 µl	5 µl*
Enzyme (100 µl)	2 µl	2 µl	2 µl
	50 µl	50 µl	62 µl

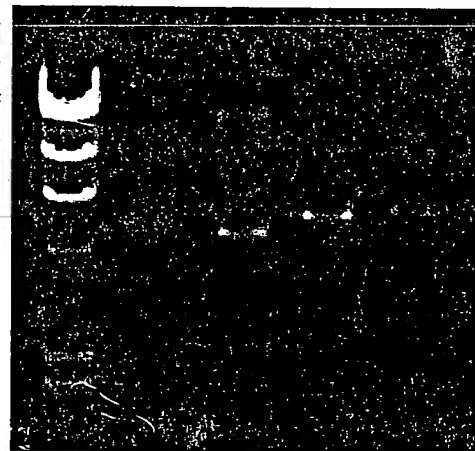
* Jung recommended this volume - conc unknown
Digest overnight at 37°C.

- * All digests went to completion.
- pVet + the GmSF prep have some contaminating genomic DNA.

- extract once (1:1) w/ phenol:CHCl₃
- extract once (1:1) w/ CHCl₃:Tropamine
- add 1/10 volume 3M NaOAc and 2.5 volumes absolute EtOH
- Precipitate DNA at -20°C

0.25 µg
Tropamine

Spe of each digest
pVet GmSF hSTNRT



The next day:

- Spin in microfuge - 15 min at 4°C
- Wash pellet in 200 µl of 70% EtOH
- Spin in microfuge - 5 min at RT 4°C

* 10% agarose gel

4/29/99

Digest #2 with Bsp I

sterile H₂O 43 μ l
 10x Buffer 4 5 μ l
 DNA pellet
 Enzyme (10u/ μ l) 2 μ l
 50 μ l

Digest 2 hours at 37°C.

Run 2% agarose prep gel - 5 lanes/double digest (Bsp I + hstNFI)
 (volume capacity = 65 μ l) \therefore Add 12 μ l of 5x blue juice = 62 μ l/digest.

By cutting 1 μ g of the pet 242 constructs, the most insert that we can recover is:

pet 242 + GmCSF \Rightarrow 5310 bp + 450 bp + 196 bp (extra n.t. from XbaI/Bsp I)
 \therefore 646 / 5456 = 11.8% \Rightarrow 108 ng

pet 242 + hstNFI \Rightarrow 5310 bp + 500 bp + 196 bp
 \therefore 696 / 6006 = 11.5% \Rightarrow 115 ng

Estimate from prep gel \approx 75 ng - 100 ng total.

- Excised the DNA-containing bands from the gel.
- GeneClean per protocol - elute in 2 x 5 μ l of sterile H₂O.

- Join the pTet vectors:

- + extract 1x lath in phenol/chloroform and chloroform/isopropanol.
- + Precipitate at R.T. w/ 1/10 vol NaOAc (3M) and 2 volumes isopropanol.
- for 1 hour - Spin down at 4°C - Wash w/ 70% EtOH

Estimate = 300 ng \rightarrow Resuspend in 12 μ l sterile H₂O = 25 ng/ μ l

Ligation Reaction:

Ratio of Insert: Vector

4485 bp - pTet vector DNA (50 ng)
 Insert DNA
 5x ligase buffer
 sterile H₂O
 T4 DNA Ligase (1U/ μ l)

2:1	4:1
2 μ l	2 μ l
3 μ l	6 μ l
2 μ l	2 μ l
2 μ l	-
110	1 μ l

GmCSF = 646 - 15 - 4485 = 7.2
 hstNFI = 696 - 15 - 4485 = 7.2

Incubate O/N
 at 15°C
 (Schweizer's lab)

4/30/49

After the ligation, precipitated DNA with 1µl 3M NaOAc, 25µl absolute EtOH, and 0.5µl yeast tRNA at -70°C for 1 hour.

* note: the resistance was set to 1000Ω instead of 200Ω so time constant on the BmsCF ligation at 2:1 was 17.4 sec.

once corrected, the time constants were 4.5 sec.

Electroporate into xLI-Blue (maintenance strains).

Recover 1 hour at 37°C in 1ml LB. Dilute 1/50 and plate on LB + 25µg/ml amp. (* note: pet 156 is amp resistant, not kan resistant like pet 21a)

5/1/99

Pick individual colonies for PCR amplification - Drop tip into 20µl LB + 50µg/ml amp for O/N culture.

PCR reactions as per usual. Template is colony pick with 50pmol each of the appropriate primers.

BmsCF = mubGM 3' (144µm) + T7 (440µm)

hSTNFR1 = hSTNFR 3' (200µm) + T7 (440µm)

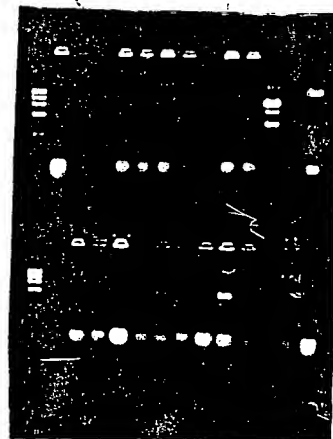
PAC1/NOT1 = PCMV (114µm) + PLNCX 3' MCS (384µm)

used zile 80 with a 60°C annealing temperature (to try to get a specific band from our PAC1/NOT1 modified PLNCX - see pg. 48)

+ 8µl of each PCR reaction on a 2% agarose gel.

* 1-5 colony picks from 2:1 ratio of insert to vector
6-10 colony picks from 1:1 ratio of insert to vector

Lane #	Sample	Lane #	Sample
1	OX174Hae II - 0.35µl	1	OX174Hae II - 0.35µl
2	BmsCF #1	2	hSTNFR1 #1
3	" #2	3	#2
4	" #3	4	#3
5	" #4	5	#4
6	" #5	6	#5
7	" #6	7	#6
8	" #7	8	#7
9	" #8	9	#8
10	" #9	10	#9
11	" #10	11	#10
12	1µl of a 1/50 dilution of pet 21a + BmsCF clone #6	12	1µl of a 1/50 dil. of pet 21a + hSTNFR1 clone #8
13	H ₂ O blank (mubGM)	13	H ₂ O blank (T7/hSTNFR1 3')
14	1µl of a 1/50 dilution of PAC1/NOT1 PLNCX (stock = 40µg/ml)	14	H ₂ O blank of PCMV + PLNCX 3' MCS



* no positives from BmsCF clones

#6 + #9 are positive for hSTNFR1 clones

5/3

- Dilute reservoir 1/100 into 3ml on cultures for pVet + GMSF → ^{PCR for} recombinant
- Did Qiagen mini-preps on the pVet + hSTARS clones #6 and #9.

Verify that XbaI and BspI sites were regenerated by the ligation

Digest #1:

sterile H ₂ O	11 μ l
10x REACT 2	2 μ l
DNA	5 μ l
Xba I (10 ⁴ U/ μ l)	2 μ l
	20 μ l

Digest 1 hour at 37°C
Phenol/Chloroform and
Chloroform/isopropanol extractions
* Take out 8 µl to run on gel
The rest to digest #2

Digest #2

Sterile H_2O	16 μ l
10X Buffer 4	2 μ l
DNA	pellet
Bsp I (100U/ μ l)	<u>2 μl</u>
	20 μ l

Digest 1 hour at 37°C
Run on gel 12% agarose

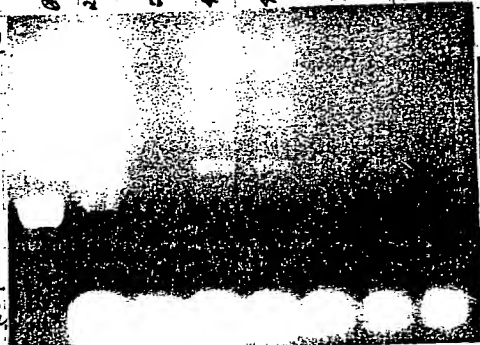
Loaded 2 μ l of single digests and 8 μ l of double digests on a 2% agarose gel \rightarrow no bands!
 Forget that this is a very low copy number plasmid.
 Repeat digests with 1 μ l of DNA (1 μ l Buffer, 1 μ l enzyme) and load as much as possible on the gel. Bag the double digests, just confirm that XbaI + BspI sites were restored and that the recombinant plasmid is of the correct size. \downarrow

 $\frac{5}{4}$

3/4
Did Diagen preps of the GMSF/purified ligation residues
for any detectable recombinants.

Reaction

sterile H ₂ O	24 μ l
dNTPs	32 μ l
10X PER Buffer II	40 μ l
10x MgCl ₂	40 μ l
MuMm 3' (144 μ m)	2.78 μ l
T7 (440 μ m)	0.9 μ l
template (diluted plasmid)	1 μ l
20x (0.5 μ l rxn)	50 μ l



Slide 83 - Add Mg - Slide 86 (94°C, 100°C, 72°C)

* from Mayra's notes the ARMISTICE is
~ 500bp - w/ the app. 100bp the hand
showed the little ??

• Allo. Anticorination & AMASE in H₂O blank. but the 2:1 (%) turn showed great.

751-1

Went back to Wayne's notebook - difficult to determine the size of the BamHI insert in pet24a, but my constructs seem to be shifted to the extent they should be on the gel - His ran a little above the 310 bp standard and mine ran a little below the 603 bp standard. \therefore size is okay.

- Could not see the recombinant plasmid band on the gel, just the non-recombinant plasmid band and it was very weak.

5/10/99

Dilute 2:1 rescue 1:100 in LB + 50 μ g/ml (same culture) to do another Diagen prep. - use 500 tip and follow the protocol for "very low copy number" plasmids.

5/11/99

Resuspend final pellet in 50 μ l of TE pH 8.

5/12/99

Resolve plasmids on a 0.8% agarose gel. Load entire 50 μ l on prep gel.

Rechecked for ϕ of plasmid⁽⁷¹⁴⁰⁾, but there was no distinction between a recombinant species and the non-recombinant plasmid - just a large smear.

Go back and cut more pet24a + BamHI and religate

Digest #1

sterile H ₂ O	54 μ l
DNA	30 μ l
10x React 2 Buffer	10 μ l
XbaI (10U/ μ l)	6 μ l
	<u>100 μl</u>

digest at 37°C for 4 hours
Run 3 μ l of digest on a 1% agarose gel -
About 85% linear. Add an additional
2 μ l of enzyme + digest overnight.

Digest #2

sterile H ₂ O	84 μ l
DNA	pellet
10x Buffer 4	10 μ l
BspI (11U/ μ l)	6 μ l
	<u>100 μl</u>

digest at 37°C
overnight

5/13/99

The next day -

POI CH₂CH Extract

CH₂CH Extract

EtOH precipitate \rightarrow digest #2 \nearrow

5/14/99 - pVet/GMCSF Cloning (cont)

Ligations set up again like those on pg. 51 using 50 ng of vector.

Inoculate o/n at 4°C.

5/15/99

Work ligations out of H₂O bath & stored frozen.

5/16/99

Precipitate ligated DNA as per usual
electroporate into XLI-Blue.

Plate 1150 on LB + 50 µg/ml Amp.

Diluted rescue into 3 ml LB/Amp 1100 as well (back-ups)

5/18/99

Pick individual colonies for PCR - Drop tip into 2 ml o/n culture.
Picks 1-10 are from 2:1 Rescue and picks 11-20 are from 4:1 Rescue.

Master Mix (1150 µl)

sterile H ₂ O	805.4 µl
dNTPs	92 µl
10x PCR Buffer II	115 µl
10x MgCl ₂	115 µl
*muM 3' #2 (135 µM)	8.5 µl
T7 (440 µM)	2.6 µl
Tag	11.5 µl
	1150 µl

* Template is a colony pick
except for the positive control
(pet24a + GMCSF) which is
1 µl of a 1/50 dilution of the
Qiagen-prepped DNA.

Ran out of muM 3' - used muM 3' #2 instead

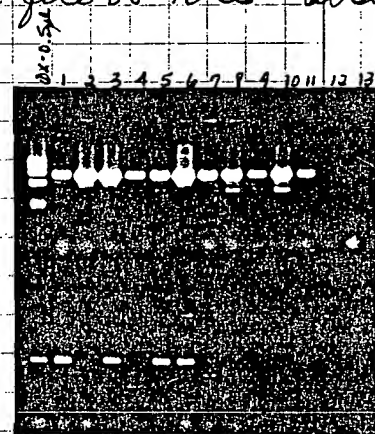
File 83 → File 86 (94, 55, 72) → File 88 (1 cycle = 94, 60 (1 min), 72 (10 min))

* Next time, change the annealing in file 88 to 55° also.

* Should have run primer alone control.

Putative Positives: 5, 8, 9, 10, 19

We saw this ~ 800bp band previously
with the T7/muM 3' primers combo.
Do Diagen prep on the putative
positives and confirm by digestion
with XbaI and BspI



14 15 16 17 18 19 20 21 22

2% Agarose gel

Cut 10 μ l of each plasmid with Xba I or Bsp I -
Reaction was 10 μ l DNA + 1 μ l 10x Buffer + 1 μ l enzyme.
Digest at 37°C for 2 hours. Load entire digest +
2 μ l of 5x loading dye on 0.8% Agarose gel.

#19 - b6 I
#10 - b6 I
I 4 - b6 I
I 4 - b6 I
#5 - b6 I
Pvt - b6 I
PVt + b6 I
#9 - x6a I
#10 - x6a I
I 4 - x6a I
I 8 - x6a I
TQX-S*
+SSNG + JWD
IWR-JWD
JPMK-WF-0

Set up 20 μ l digests of each plasmid:
 18 μ l of DNA (GmSF) or 15 μ l of DNA (HSTNPTI) + 3 μ l sterile H_2O
 2 μ l of 10x Buffer
 2 μ l of enzyme

Digest with *Xba*I just - o/n at 37°C
next day:

Set up 2nd digest as above (but in 10 μ l)

Run total digest on 2% agarose gel.
Could not see an insert on any of the recombinants,
but the vector backbones were probably only about
100ng bands. — Go for the transformation into
the host strain.

6/6-12/99

6/6/99 Purification of rHSTNFEI (under non-denaturing conditions)

- Took the 1ml of soluble cytosolic rHSTNFEI and ran on His-Binding resin according to the protocol. Filtered the sample with a 0.45µm filter before applying to column.
- Washed the column as described and eluted in 1x elution buffer - collected 1ml fractions.
- Stripped column w/ 1x strip buffer and collected 10 x 1ml fractions
- Ran 31.5 µl ~~reduced~~ reduced & unreduced on a 17.5% SDS/PAGE gel - saw no bands.

*Potential problem with the 1.5M His pH 8.8 used for the resolving gel

6/7/99

- made a 1L culture to make new prep
- Processed as described on pg. 57 - did 2 extractions ^{one w/ 50µl 2.5M} with an additional 25ml of merc-D buffer. It was difficult to determine if the lysozyme was effective after the first extraction, so we elected to do a second. Add ~~25~~ additional lysozyme (100 µg/ml) and 3 freeze/thaw cycles as before. Spin at 27,000 xg for 30 min.
- Filtered the extractions separately through a 0.45µm filter - about the same viscosity. Combined the two, added 0.05% NaN₃ and ran over a charged His-bind resin. overnight ~ 24 hours total.
- Washed and eluted as described (in 1M imidazole).
- Tested fractions in BCA assay.

* diluted sample 1:1 with binding buffer + 0.05% NaN₃

Blank = 200mM imidazole elution buffer. ^{Changed the working reagent to ~~total~~ ^{immediately}}
Dilutions of fractions were made by diluting the neat sample 1/5 in dH₂O, and then the 1/5 sample 1/2 in 200mM imidazole elution buffer.

BSA	neat	1/5	1/10
10	FR1		
20	FR2		
40	FR3		
80	FR4		
160	FR5		
320	FR6		
640			
1280			

Regression Line:

$$y = -3.8522e-2 + 1.0692e-3x \quad r^2 = 1.0$$

Estimate of protein concentration:

FR #1: neat = 100 µg/ml

FR #2: neat = 132 µg/ml

1/5 = 189 µg/ml

$\bar{x} = 161 \mu\text{g/ml}$

6/10 silver stain of SDS/PAGE Gel - Purified rhTNFRI
 - 20 lanes relative purity & size

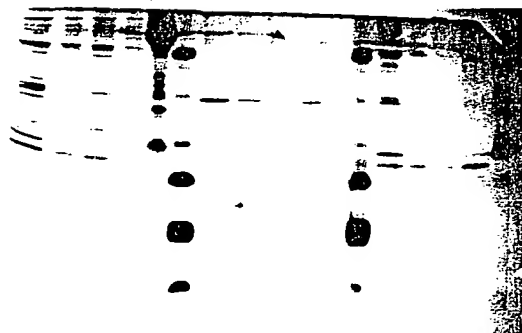
Lane	Sample
1	FR #2 - 5 μ g
2	FR #2 - 1 μ g
3	FR #1 - 5 μ g
4	FR #1 - 1 μ g
5	TNFBPI(R+D) - 0.9 μ g
6	low MW markers - 2.5 μ g
7	JDE'S 4B2 SC Ab - 5 μ g
8	JDE'S 4B2 SC Ab - 1 μ g
9	" " " " - 5 μ g
10	" " " " - 1 μ g
11	low MW markers - 2.5 μ g
12	FR #2 - 5 μ g
13	FR #2 - 1 μ g
14	FR #1 - 1 μ g
15	FR #1 - 5 μ g
16	TNFBPI(R+D) - 0.9 μ g

reduced

unreduced

* quantities loaded represent the concentrations estimated from BEA assay. They apply to the way off.

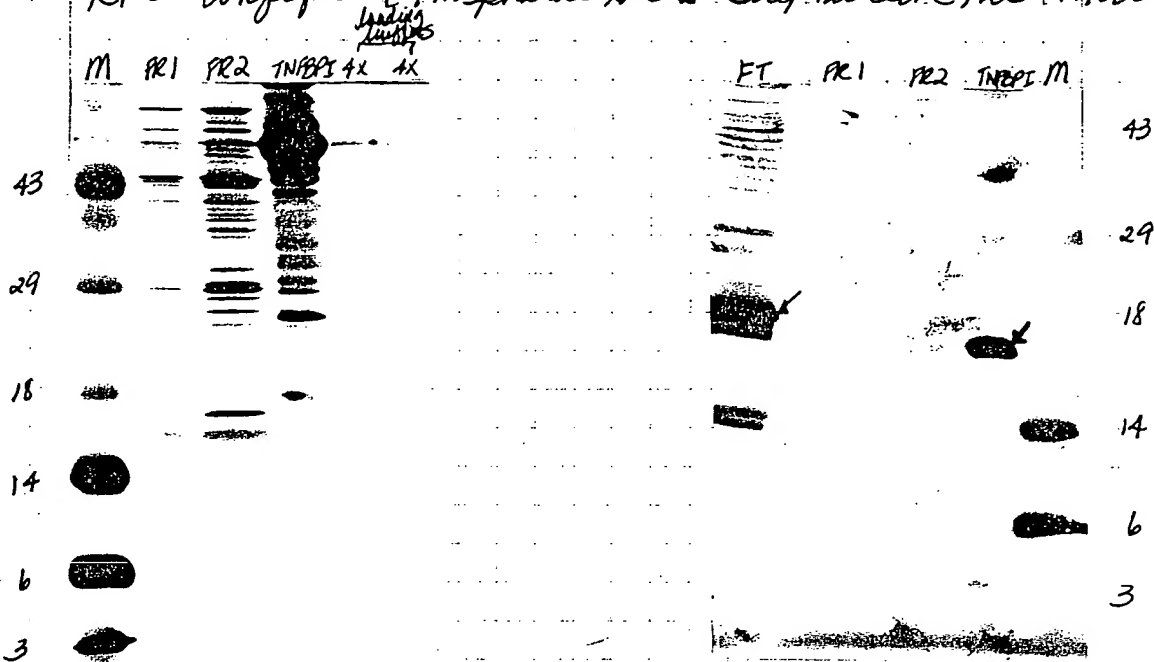
* 17.5% SDS/PAGE gel
 stained 15 min. in silver nitrate.



to the 4x buffers with BME contaminated. All the samples on the left are in this buffer except for the markers and gels samples (they were diluted in 2x buffers). Fraction #2 in the buffer w/o BME (lane #12) appears fairly pure, but the corresponding sample in the buffer w/ BME (lane #13) has a lot of other proteins. Perhaps the BME is reducing the jaw proteins that are evident in lane #12 to multiple species of varying sizes. The protein that is our putative rhTNFRI is running a little smaller than predicted (estimated MW = 21,057 Da). Need to run a Western to confirm that we have this protein purified via His-tag and that it is of the correct size. The samples are in middle, so their relative migration in the gel will be ~~un~~ altered.

6/12

17.5% resolving gel - 3% stacking gel
 Loaded 25 μ l of each test sample w/ 25 μ l loading buffer + BME.
 (including the 'R+D systems' TNFBI $\therefore \sim 0.625 \mu$ g).
 Markers are 2.5 μ g for silver stain and 7.5 μ g for Western
 loaded 4x loading buffer at 12.5 μ l + 37.5 μ l H_2O .
 KPL conjugate (phosphatase labeled streptavidin) at 1:1000



* 4x loading buffer is
 not contaminated with
 protein.

FT = slow through from His Column

The bands on the Western marked with arrows (✓) came up within
 10-20 seconds. Most of our protein appears to be in the
 "slow through" suggesting that it did not bind the His Column.
 There may be a slight hint of protein in PR1 and PR2 -
 but the amount is very small (band running at ~ 15 kDa).
 Perhaps the His tag is inaccessible due to the secondary
 structure of the protein. Because the expression cassette
 was isolated from the pET24A construct that we know has
 the tag, the possibility that the His tag is not made
 with this expression system seems remote. We
 may want to try to purify the protein via an
 antibody to the N-terminal tag. There
 does appear to be a lot of rTNFBI that was solubilized,
 but that remained in the slow through

7/7/99

7/7 Purification of rHSTNFR1 - Strep tag (Affinity Purification via Streptactin Affinity Column)

1 mM IPTG
induction at 37°C for 3 hours

* Made periplasm from some of induced cultures as described (under protocol)

* Ran all steps at room temp.

* Did not purify clone 19 periplasm

Procedure

- 1) Remove top then bottom caps and allow storage buffer to drain
- 2) Equilibrate the column with 5 ml of buffer W (0.1M His pH 8, 1M NaCl, 0.05% NaN₃)
- 3) Add periplasm to column. Let it run through completely.
- 4) Wash the column 5x with 1 ml of buffer W. Collect the eluate in 1 ml fractions.
- 5) Elute the recombinant protein with 6x 0.5 ml buffer E (buffer W + 2.5 mM desthiostatin). Collect the eluate in 0.5 ml fractions.
- 6) Regenerate the column by washing 3x with 5 ml buffer R (buffer W + 1 mM HABA). The color change from yellow to red indicates regeneration - the intensity of the red is an indicator of column activity.
- 7) Add 2 x 4 ml of buffer W. Store column at 20°C with 2 ml of buffer W.

* Assume extinction coefficient is 1.5 (as published)

Clone 7 = 0.0858 mg/20 ml \Rightarrow 4.29 mg/L

Clone 10 = 0.0198 mg/20 ml \Rightarrow 1 mg/L

	A ₂₈₀ mg/ml
Blank	0.0008
Clone 7	-0.0139E1
	-0.0114E2
	0.0915E3
	0.0305E4
	0.0067E5
Clone 10	-0.0025E6
	0.0083E1
	-0.0149E2
	0.0089E3
	0.0208E4
	-0.0140E5
	-0.0164E6

Test Purified Fractions in standard ELISA

40	1.873	1.716
20	1.452	1.425
10	1.155	1.059
5	0.748	0.684
2.5	0.424	0.404
1.25	0.245	0.241

Ran standard curve (RTD Systems rHSTNFR1) at 1.25 - 40 ng/ml diluted in elution buffer

Ran the washes from each periplasm purification (i.e., 5 each)

at 1/100 and the elution #3 from clone 7 and the elution #4 from clone 10 at 1/100 - 1/100,000 - Periplasm from clone 19 at 1,000 - 1/100,000.

NO SIGNAL FOR TEST WELLS

* TFW? I used the concentration of the combined fractions to determine the dilutions to use in the ELISA (i.e., 80 µg/ml and 19.8 µg/ml) - these are too dilute?

* Standard curve did give a signal for 1.25 ng/ml \rightarrow Clone #7 at E3

8/9/99

8/9 ~~ELISA~~ for rhTNFRI Binding to TNF- α - Streptag Detection

We need to confirm that the strep tag is accessible. Therefore, we will repeat the previous assay to confirm the binding of the rhTNFRI to TNF as detected by SA-AP binding to the strep tag.

Procedure

- 1) Coat plate with 2 μ g/ml human TNF- α .
 - 2) Add periplasms at 1:10 or 1:100 diluted in 0.1% BSA/PBS (want to dilute out the sucrose from the buffers P).
 - 3) Add KPL SA-AP at 1:1000
- Incubation = 15 min at RT (no signal) then DN at 40C.

	1	2	3	4	5
A	3 hours 0.05 1/100	0.1 1/100	1 1/10	Azurin 1/10	B 1/100
B	3 hours 0.05 1/100	0.1 1/100	1 1/100	0.1% BSA/PBS Tween	Buffer P
C	6 hours 0.05 1/100	0.1 1/100	1 1/100		
D	1/100	1/100	1/100		
E	9 hours 0.05 1/100	0.1 1/100	1 1/100		
F	1/100	1/100	1/100		
G	24 hours 0.05 1/100	0.1 1/100	1 1/100		
H	1/100	1/100	1/100		

Absorbance Report Single Wavelength

Blank
Mean. 0.249
Std.Dev. 0.000

	1	2	3
A	-0.042	-0.017	-0.001
B	-0.043	-0.045	-0.044
C	-0.037	-0.030	0.303
D	-0.024	-0.050	0.053
E	-0.028	0.142	0.206
F	-0.041	-0.021	0.009
G	-0.045	-0.054	0.030
H	-0.057	-0.030	-0.017

	4	5
A	0.000	-0.024
B	-0.040	-0.052

detection)

rhTNFRI

Positives: 6 hours at 1mM and 9 hours at 0.1mM and 1mM. This data coincides w/ the previous assay. These samples were the highest as detected by the goat α rhTNFRI Ab as well.

Like in the Western blot of 7/28 (pg 74), the binding of streptavidin to the strep tag is weak. Perhaps the protein is folded such that the tag is inaccessible. This may prove to be a major problem if we choose to surface the protein on the streptacin column.

TNF- α
10% w/v
made
IPTG
100x

9/1-8/99

clone #7

9/1 Expression + Purification of rNSTNFR1 from pAK401NSTNFR1 clone 7

We are now going to attempt to isolate the rNSTNFR1 from clone 7 since it represents the native, non-mutated form of the protein.

Procedure for Periplasmic Preparation

- 1) Subcultured a single colony of clone 7 onto a fresh LB plate + 50 µg/ml amp. - grow at 37°C
- 2) Picked a single colony and inoculated a 25ml overnight of LB + amp - grow at 37°C
- 3) The next day, dilute overnight 1:50 into 1L of LB + amp and grow to an OD₅₅₀ = 0.5 - grow at 37°C
- 4) Induce expression with 1mM IPTG. Grow an additional 6 hours at 37°C.
- 5) Harvest the bacteria at 4500 x g for 15 min at 4°C. Save the culture supernatant.
- 6) Resuspend the pellets in a total of 5ml of Buffer P (note: should have used some according to the protocol).
- 7) Place on ice for 3 min.
- 8) Centrifuge at 16,000 x g for 15 min at 4°C. Remove periplasm to a fresh tube. Filter through a 0.2 µm filter before running on the column.

9/3

Procedure for Strip Column purification

- * Followed the manual for all procedures except the column was eluted with 7 x 0.5ml of Buffer E (desthiotriton buffer).

Assayed the washes and elutions by BCA. Only the first two washes had detectable protein. This protein likely is non-specific bacterial proteins. No protein detectable in the elutions. Is the strip tag inaccessible? From the sequence data we know that the tag is being made. We need to characterize the production of NSTNFR1 from clone 7 further.

9/8 ELISA of Clone 7 Periplasm Binding to TNF

Standard protocol:

- 1) Coat plate with 2 μ g/ml of TNF from either PeproTech (to be used to make the affinity column) or Chemicon. Incubate on at 4°C.
- 2) Block plate w/ 200 μ l of 2% BSA in PBS.
- 3) Add test sample, control, or standard (100 μ l/well) diluted in 0.1% BSA/PBS/ Tween * wanted to dilute out the sucrose in the buffer P-containing samples.
- 4) Add SA-AP at 1:1000 (KPL)
- 5) Add PNPP substrate.
R+D STNPR1 on Chemicon TNF = 10 min - the rest were 20 min.

Samples (notes)

- 1) The "flow through" is that which was run through the Strep Column and collected.
- 2) E. coli supt = the supernatant from a culture that you grew - this serves as a negative control for testing for any STNPR1 in the culture supt. of Clone 7 (once the bugs were spun out)
* Mark found a paper where the authors isolated recombinant single chain antibodies from the supernatant rather than the periplasm. They did an Amsox cut and then dialyzed and purified their protein. We just wanted to know if any STNPR1 is being secreted by Clone 7.
- 3) Azurin periplasm = the positive control that Biometra sent us a long time ago for use in assays determining strep tag binding \rightarrow it is Azurin + the strep tag attached. It serves as a periplasm negative control here.

* well 4C got Clone 7 periplasm by mistake.

TNF

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	R+D STNPRE ↓ 1	Clone periplasm ↓ 2	R+D STNPRE ↓ 3	Superon clone culture ↓ 4	AS ↓ 5
A	Peptidase TNF 2.5mg/ml	1/2	Chemical TNF 2.5mg/ml	1/2	1/8
B	5	1/4	5	1/4	slow growth 1/2
C	10	1/8	10	X	1/4
D	20	Buffer p	20	Buffer p	1/8
E	40	Oxolin periplasm 1/10	40	Oxolin periplasm 1/10	Escherichia sup (control)

Absorbance Report
Single Wavelength

Blank
Mean. 0.295
Std.Dev. 0.000

	1	2	3	4	5
A	-0.179	0.211	0.084	0.034	0.014
B	-0.166	0.073	0.271	0.008	0.311
C	-0.137	-0.034	0.355	0.055	0.155
D	-0.093	-0.181	1.936	0.000	0.099
E	-0.072	-0.178	***	-0.014	-0.010

Raw Data Report
Single Wavelength

	1	2	3	4	5
A	0.116	0.506	0.379	0.329	0.309
B	0.129	0.368	0.566	0.303	0.606
C	0.158	0.261	1.150	0.350	0.450
D	0.202	0.114	2.231	0.295	0.394
E	0.223	0.117	***	0.281	0.285

This is the first real evidence that we have to support the notion that STNPRE is being secreted into the periplasm in a biologically active form (i.e. it binds to TNF). This binds both Peptidase's & Chemical TNF.

We should probably do the induction optimization studies on Clone 7 like we did for clone 10. This includes varying the temperature of growth and the duration of the IPTG induction. We will start with a concentration of 1mM IPTG.

* Note: The R+D System's STNPRE does not bind to the Peptidase TNF.

9/8 Silver Stain + Immunoblot of Clone 7 Periplasm

- 17.5% resolving gel run on → Ran very slowly. So there a problem with the sucrose in the Buffer P?
- Loaded: 5 μ l of markers (low MW) for the silver stain and 37.5 μ l for the Western blot; loaded 37.5 μ l of the R+D protein (rhSTXPI); 37.5 μ l of the Clone 7 periplasm; 10 μ l of the Azurin periplasm; and 37.5 μ l of the flow through of Clone 7 periplasm (through the strep tag column).
- * The silver stain gel was mw markers, the flow through & the R+D rhSTXPI (did not have enough of the starting Clone 7 periplasm to run).

Result: no proteins were visible in the 14-18 kDa range of the flow through. The R+D protein stained as per usual.

Immunoblot

Panel A
mw markers
flow through
Azurin & control

Panel B
mw markers
Clone 7 periplasm
flow through
R+D rhSTXPI

Incubation with
substrate = 10 min.

room
def.
21
The results are consistent with the ELISA data. Indeed, the rHSTNFR1 being produced by ~~most~~ clone 7 is being secreted in to the periplasm where it is likely being folded properly (hence it binds to TNF).

tag
w
enough
-18KDa stained
The problem, however, is that we could not purify this material ~~on~~ on the strep column. No protein was detected in the elutions assayed by BCA. Perhaps the levels in these samples are below the lower limits (i.e. 50 µg/ml) of the BCA assay. I did not save these fractions, so they cannot be assayed by ELISA. The protein was not found in the flow-through either. ~~It is still on the column.~~
We can conclude, however, that we cannot purify appreciable ~~the~~ rHSTNFR1 via the strep column under these conditions.

Fortunately, the protein being produced by clone 7 appears to be native and properly folded, so we could potentially purify it on the TNF column.

we can also try to optimize protein production and secretion into the periplasm

9/22-26/99

9/22 Depletion of STNFR1 - spiked plasma using
 • Immobilized TNF and α -STNFR1 Antibodies

We need to demonstrate that we can deplete biological fluids such as plasma/serum and/or ultrafiltrate of STNFR1 by exposing the said fluid to ~~antibodies~~ TNF or ~~antibodies~~ to STNFR1. Such depletion would justify the development of matrices modified to treat cancer patients.

Initially, we will spike human plasma (mine) with STNFR1. Since we know, based on previous assays, that my blood contains no detectable STNFR1. This plasma will then be incubated with CNBr-activated sepharose conjugated to either TNF, goat α hSTNFR1 Ab (R+D polyclonal Ab or mouse α hSTNFR1 mAb (BioSource), or an irrelevant matrix (DT145 (α V β 6 Ab)). The samples will be assayed for a reduction in STNFR1 levels relative to the original, "spiked" starting material.

Just of all, however, we need to determine if the plasma proteins are going to inhibit the assay in any way, and second, if the level of STNFR1 that we have estimated in fraction 3 of the TNF affinity column gives us a signal comparable to the R+D standard when diluted in plasma.

Assay #1

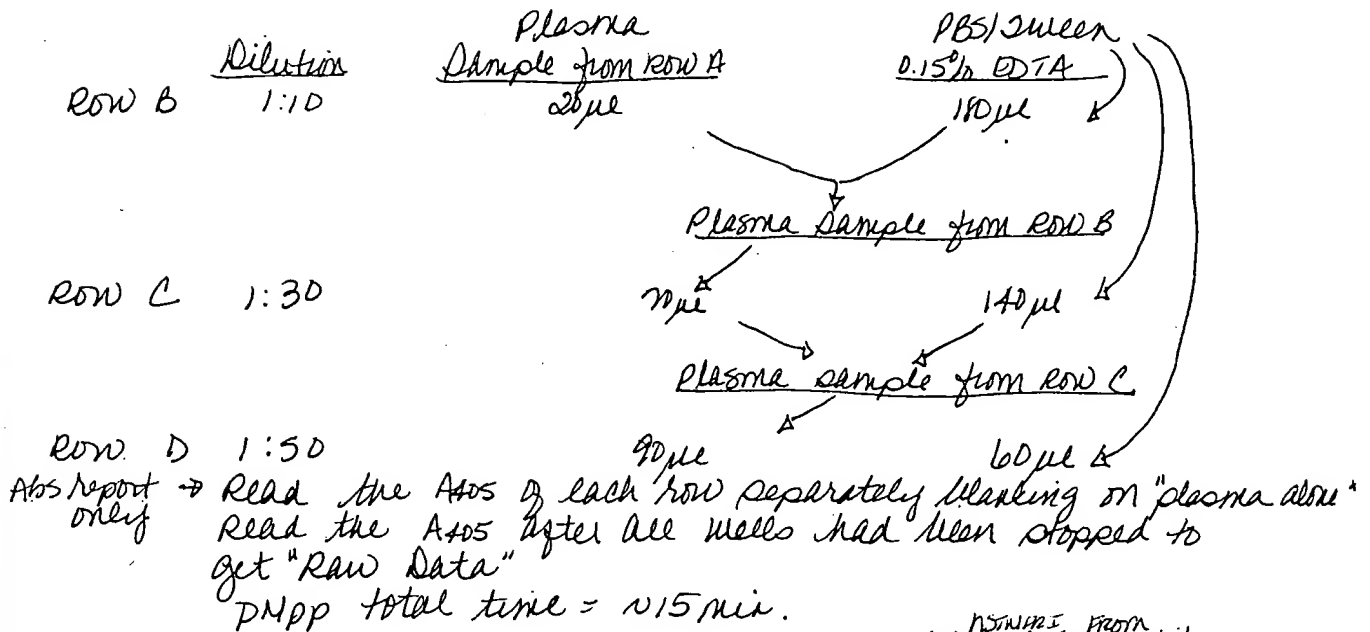
Standard STNFR1 ELISA with goat α hSTNFR1 polyclonal Ab: Capture + detection.
 or our hSTNFR1 from the Hep2 cell line.

Test samples are the R+D hSTNFR1 diluted in plasma or plasma diluted 1:10, 1:30, or 1:50 in PBS/12mM (no BSA) with 0.15% EDTA. The EDTA is at the same concentration as that in the tubes in which the blood is drawn (prevents coag). We are doing a dilution series to determine under which plasma concentration we get the best standard curve with the lowest background. All concentrations are in ng/ml - we will start our hSTNFR1 (fraction 3) at 40 ng/ml in case our estimate of concentration was inaccurate. Each row is, in essence, a separate assay that will be allowed to develop independently of the other rows. To make the dilution series, 20 ng/ml of STNFR1 is added to undiluted plasma, and then diluted in a two fold series into undiluted plasma so the total plasma

* Had 10 tubes of blood drawn \approx 50ml \rightarrow this is about 50% plasma. Spin in 3000 rpm centrifuge, letting 4-10 min. remove plasma. Spin again letting 5-10 min to get residual plasma + remove the platelets. Filter plasma through a 0.45 μ m (pore size approx) filter \rightarrow lost about half have 12.5 ml.

this is how A

From these standards, the following dilutions are made:



R+D rhSMFRI Standard

NOTE: From conc. wells supernatant in TNE aff. column - fraction 3

	1	2	3	4	5	6	7	8	9	10	11	12
1:10 A	20	10	5	2.5	1.25	0.625	0.3125	0.15625	40	20	10	plasma alone
1:30 B	2	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	4	2	1	plasma alone
1:50 C	0.66	0.33	0.166	0.0833	0.04166	0.02083	0.010416	0.005208	1.33	0.66	0.33	plasma alone
1:50 D	0.4	0.2	0.1	0.05	0.025	0.0125	0.00625	0.003125	0.8	0.4	0.2	plasma alone
E	plasma + 20 µl A405	plasma + 20 µl A405										

* E1 got everything by mistake!

Raw Data

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.981	1.828	1.781	1.691	1.566	1.485	1.482	1.474	1.876	1.615	1.670	1.388
B	1.547	1.154	0.959	0.909	0.816	0.808	0.827	0.794	1.948	1.549	1.277	1.262
C	1.111	0.773	0.574	0.522	0.475	0.443	0.420	0.418	2.202	1.397	0.947	0.847
D	0.733	0.492	0.370	0.324	0.292	0.279	0.274	0.268	1.597	0.964	0.595	0.521
E	***	0.085										

	1	2	3	4	5	6	7	8	9	10	11	12	Blank
A	0.684	0.541	0.492	0.341	0.297	0.217	0.205	0.176	0.507	0.274	0.340	0.000	Mean. 1.109
B	0.271	-0.068	-0.231	-0.279	-0.361	-0.366	-0.341	-0.403	0.599	0.293	0.039	0.000	1.097
C	0.260	-0.057	-0.230	-0.284	-0.323	-0.357	-0.376	-0.379	1.231	0.514	0.100	0.000	0.767
D	0.194	-0.014	-0.131	-0.175	-0.202	-0.213	-0.221	-0.230	0.997	0.398	0.068	0.000	0.475

made:

The "next" samples (in 100% human plasma) give ^{permi-}comparable results:

	RTD STNFKI	our Hela-derived STNFKI
40 ng/ml	-	1.876 849 → 0.507
20 ng/ml	1.981 849 → 0.684	1.651 → 0.274
10 ng/ml	1.828 → 0.541	1.670 → 0.340

We can use the plasma undiluted and just subtract out the background.

* We have decided to use our Hela STNFKI for both the standard curve and for spiking the plasma for depletion. This allows the assay to be internally controlled.

plasma alone to

fraction 3

12

plasma alone
plasma alone
plasma alone
plasma alone
plasma alone
plasma alone

88
62
47
21

Blank

1.109
1.097
0.767
0.475

9/26/99 Assay #2 - Depletion of STNFKI from Plasma

Procedure

- 1) Took ^{4.5 ml} plasma and spiked it to 20 ng/ml STNFKI (with the protein purified on the TIE column - fraction #3)
- 2) Took out 0.5 ml of this spiked plasma to make standard curve. Made 2-fold dilutions in unspiked plasma (creat).
- 3) To the remaining 4 ml, 4 ml of unspiked plasma was added to yield 40 ng/ml final STNFKI concentration.
* Removed 200 µl as the "starting material". Diluted this 2-fold in PBS + Tween + 0.15% EDTA (prevent coagulation)
- 4) Took ~0.5 ml of each resin and spun on setting 3 for 10 min in the ⁴⁰⁰cc clinical centrifuge. Removed as much of the PBS/supernatant as possible and washed each in 10 ml of fresh PBS. Spin + decanted sup.
- 5) Added 1.8 ml of spiked plasma to each conical tube containing the resins, and rotated on the rotator placed in the 37°C warm-room on the 4th floor.
- 6) After 15 min., the tubes were placed upright, the beads allowed to settle, and 0.4 ml of supernatant was removed.
- 7) The residual beads were removed by centrifugation on setting 5 (~2000 x g) on the microfuge (5000 rpm). The sup. was removed and diluted 1:1 with PBS + Tween + 0.15% EDTA.

9/26

- 8) Repeat 6+7 at 30, 45, and 60 minutes post head addition.
- 9) Add all samples to the ELISA plate coated with goat α hSTNPR1 Ab and blocked with 2% BSA.
- 10) Complete ELISA as per usual.

DNpp Incubation = ~5min. at RT.

	1	2	3	4	5	6	7	8	9	10	11	12
A	20	10	5	2.5	1.25	0.625	0.3125	0.15625	plasma alone	spiked starting material A/GT	1/2	1/4
15min B	Depletion w/ TNF N	→ 1/2	Depletion w/ poly Ab N	→ 1/2	Depletion w/ irrelevant protein N	→ 1/2	Depletion w/ mAb N	→ 1/2				plasma + SA, AP along with 20
30min C												
45min D												
60min E	↓	↓	↓	↓	↓	↓	↓	↓				

Raw Data Report Single Wavelength

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.874	1.867	1.724	1.622	1.508	1.513	1.338	1.370	1.459	1.604	1.347	1.071
B	1.493	1.180	0.245	0.209	1.279	1.046	1.061	0.791	0.040	0.041	0.038	0.090
C	1.563	1.351	0.209	0.174	1.230	1.069	1.029	0.779	0.042	0.042	0.042	0.029
D	1.562	1.371	0.174	0.150	1.348	0.102	1.048	0.735	0.050	0.045	0.042	0.053
E	1.479	1.207	0.169	0.151	1.271	0.958	1.002	0.745	0.038	0.047	0.050	0.054

Regression line of standard curve (1.25 - 20ng/ml):

$$y = 3.3148e-2 + 0.32455 \log x \quad r^2 = 0.956$$

Absorbance Report Single Wavelength

Blank
Mean. 1.459
Std.Dev. 0.000

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.415	0.408	0.265	0.163	0.049	0.004	-0.121	-0.087	0.000	0.145	-0.112	-0.388
B	0.034	-0.279	-1.214	-1.250	-0.180	-0.413	-0.398	-0.668	-1.419	-1.418	-1.421	-1.369
C	0.104	-0.108	-1.250	-1.285	-0.229	-0.390	-0.430	-0.680	-1.417	-1.417	-1.417	-1.430
D	0.103	-0.088	-1.285	-1.309	-0.111	-1.357	-0.411	-0.724	-1.409	-1.414	-1.417	-1.406
E	0.020	-0.252	-1.290	-1.308	-0.188	-0.501	-0.457	-0.714	-1.421	-1.412	-1.409	-1.405

* Clearly, there is some STNPR1 in my plasma that is being detected in the ELISA (well A/G raw data). Nonetheless, the STNPR1 levels in my plasma spiked with fraction 3 STNPR1 were partially depleted by the mAb and Donalidol, detected but the

9/26

We need to confirm those results. There also seemed to be a reduction in the overall concentration of STNFR1 upon the plasma was incubated with the "irrelevant matrix" (α -V β Ab). This could reflect merely a dilution effect by adding 0.5 ml of beads plus some residual PBS. We need to control for this variable as well. Of some concern is the fact that the TNF-conjugated beads did nothing to reduce the level of STNFR1. Is this column dead by virtue of coupling the TNF to CNBr-activated Sepharose? We will repeat the assay with fresh plasma. Because there is endogenous STNFR1 in my plasma, there is really no need to quantify the levels pre and post-treatment, but rather to define the overall depletion of STNFR1 by incubating the plasma with the various matrices. We will define t=0 by:

- mixing the beads with plasma on ice
- spinning the sample immediately to remove the beads
- take sample from the supernat.

* This will account for the dilution effect of the beads.

* For this assay, use 1 ml of plasma / 0.25 ml of beads. The rest of the procedure is the same.

	1	2	3	4	5	6
A	Spiked 1		Spiked 1	Spiked 1	Spiked 1	Spiked 1
B	Spiked 1		Spiked 1	Spiked 1	Spiked 1	Spiked 1
C	Spiked 1		Spiked 1	Spiked 1	Spiked 1	Spiked 1
D	Spiked 1		Spiked 1	Spiked 1	Spiked 1	Spiked 1

Raw Data Report		Absorbance Report					
Dual Wavelength		Dual Wavelength					
		Blank					
		Mean. 0.314					
		Std. Dev. 0.000					
	1	2	3	4	5	6	
A	0.957	-0.303	0.939	0.843	0.608	0.664	
B	0.769	0.000	0.919	-0.198	0.368	0.701	
C	-0.303	-0.305	0.790	-0.201	0.984	0.439	
D	-0.304	-0.305	0.997	-0.205	0.441	0.805	

* note: I loaded the samples in row C incorrectly:

3C is actually the 30 min. sample for the irrelevant matrix;
 4C is " " " " " " monoclonal Ab matrix;
 And 5C " " " " " " TNF matrix.
 4C is correct.

* The "SA-AP - no 2nd Ab" control accidentally got 2nd for about 2 min. I realized it and washed the well w/ PBS/ Tween and incubated it with: PBS + 0.1% BSA + 0.05% Tween.

with

12

14

25MA+
APD
CAG
50

3
3
3
5
5

class,
3 STNFR1
ed but the